J. Clin. Chem. Clin. Biochem.
Vol. 21, 1983, pp. 129–132

Serum Total Hydroxyproline Assay: Effects of Age, Sex and *Paget*'s Bone Disease

By *T. J. Gilbertson*

Medical Development and Clinical Laboratory Research, The Upjohn Company, Kalamazoo, Michigan,

M. N. Brunden

Biostatistics, The Upjohn Company, Kalamazoo, Michigan,

S. B. Gruszczyk

The Clinical Research Laboratory, The Upjohn Company, Kalamazoo, Michigan,

M. P. Whyte

Division of Bone and Mineral Metabolism, Washington University School of Medicine, St. Louis, Missouri and

M. A. Burnett

The Clinical Research Laboratory, The Upjohn Company, Kalamazoo, Michigan

(Received May 7/September 14, 1982)

Summary: An assay was developed for total hydroxyproline (protein, peptide and free) in 12 h fasted human serum. Values obtained from 80 adults judged normal by laboratory and physical examinations showed age and sex effects; values increased with age and were higher for men. Assay of serum samples from 22 patients with *Paget*'s bone disease revealed values significantly above normal taking age and sex into consideration ($p = 0.001$). Serum alkaline phosphatase and serum hydroxyproline values for these patients were correlated ($r = 0.91$).

Serum total hydroxyproline may be a useful assay for evaluating metabolic bone diseases but age and sex must be taken into consideration in the interpretation.

Bestimmung des gesamten Hydroxyprolins im Serum: Einfluß von Alter, Geschlecht und M. Paget

Zusammenfassung: Ein Verfahren für die Bestimmung des gesamten (Protein-, Peptid- und freien) Hydroxyprolin im Serum des Menschen nach 12 h Fasten wurde entwickelt. Die Werte von 80 durch Laboratoriums- und physikalischen Untersuchungen als normal befundenen Erwachsenen zeigten Alters- und Geschlechtsabhängigkeit: Die Werte stiegen mit dem Alter an und waren für Männer höher. Unter Berücksichtigung von Alter und Geschlecht waren die Hydroxyprolinkonzentrationen im Serum von 22 Patienten mit M. *Paget* signifikant ($p = 0{,}001$) höher als beim Referenzkollektiv. Die Korrelation zwischen alkalischer Phosphatase und Hydroxyprolin im Serum der Patienten betrug $r = 0{,}91$.

Für die Bewertung metabolischer Knochenerkrankungen kann die Bestimmung des gesamten Hydroxyprolin im Serum hilfreich sein, jedoch müssen bei der Interpretation Alter und Geschlecht berücksichtigt werden.

0340-076X/83/0021-0129$02.00

Introduction

Hydroxyproline is formed by the hydroxylation of proline after its incorporation into protein during collagen biosynthesis (1). Hydroxyproline, hydroxyproline-containing peptides and hydroxyproline-containing proteins occur in serum and urine as the results of collagen metabolism. Hydroxyproline does not appear to be reused for protein biosynthesis (1). Assay of circulating hydroxyproline levels should be a useful indicator of collagen catabolism.

Since dietary hydroxyproline readily enters the serum and urine, current urinary assays require that people be fasted or on low collagen diets to obtain assay values which reflect endogenous collagen metabolism (2). Development of a serum assay might allow the acquisition of meaningful values with the less rigorous patient preparation of a 12 h fast.

Assays have been reported for the various fractions of serum or plasma hydroxyproline (2). In our opinion, none of them have been shown to give more useful biological or clinical information than that to be expected for a total hydroxyproline value.

Since we wished to be able to process a fairly large number of samples, we modified the *Blumenkrantz & Asboe-Hansen* method (3) for total urinary hydroxyproline to serum.

Materials and Methods

Apparatus

A Modular Dri-Bath, Sybron/Thermolyne (Thermolyne Corporation, Dubuque, Iowa 52001) was used for sample hydrolysis.

We used an Autoanalyzer I system for the assay as previously described (3).

Reagents

Hydrochloric acid, sodium hydroxide, citric acid, disodium phosphate and 700 g/kg perchloric acid were all analytical reagent grade from Mallinckrodt, Inc., St. Louis, Missouri 63147.

p-Dimethylaminobenzaldehyde was gold label grade from Aldrich Chemical Company, Milwaukee, Wisconsin 53233.

Chloramine T and phenolphthalein were from Eastman Kodak Company, Rochester, New York 14650.

L-Hydroxyproline was purchased from J. T. Baker Chemicals, Phillipsburg, New Jersey 08865.

Procedures

Standards

Hydroxyproline standards are prepared in distilled water to contain 3.8 to 30.5 µmol/l following hydrolysis and dilution with the pH 6 buffer.

Sample and standard hydrolysis

One milliliter of serum or standard is mixed with 1 ml of 12 mol/l HCl in a 15 ml tube sealed with a teflon-lined screw cap (a 0.5 ml sample can be used with 0.5 ml of 12 mol/l HCl to conserve sample). The tubes are placed in a dry block heater and hydrolyzed for 16 h at 100 °C. The hydrolyzates are cooled and filtered through glass wool-plugged dispo-pipettes. One half ml of 6 mol/l NaOH is added to a 0.5 ml aliquot of hydrolyzate and the mixture is cooled in an ice bath. Phenolphthalein solution (one drop of 10 g/l phenolphthalein in isopropanol) is added to each tube. NaOH (1 mol/l) is added to each tube until a pink color is obtained. The volume of the samples is adjusted to 5 ml with 0.2 mol/l citrate phosphate buffer (pH 6). The hydrolyzed, diluted, pH 6 samples and standards are analyzed for hydroxyproline with the autoanalyzer.

Autoanalyzer procedure

The autoanalyzer is set up as previously described (3). Note the following changes in the reagents: The samples and standards are alternated with distilled H_2O to reduce carry-over. The chloramine T concentration is increased to 49.6 mmol/l and the solvent is changed to ethanol/water (volumes, 1+1).

Results and Discussion

Sample preparation is essential to adjust the pH of all the samples to pH 6. The control of pH is important for reproducible results. The previous report (3) is not clear on this point.

Increased chloramine T concentration is necessary to provide sufficient oxidizing agent to react with the hydroxyproline and other reducing agents in the hydrolyzate. The ethanol/water solvent is used to allow the oxidized sample to mix easily with the *p*-dimethylaminobenzaldehyde solution.

Results of a recovery study and precision study are shown in table 1. The average recovery of 81.7% is lower than we would like. We were not able to increase this by changing the chloramine T concentration, the *p*-dimethylaminobenzaldehyde concentration or by using various solvents.

Results of the analysis of 12 h fasted serum from 80 adult humans judged normal by laboratory and physical examination are shown graphically in figures 1 and 2. These results are consistent with a linear statistical model (4) incorporating both age and sex. The overall model fit is highly significant ($p < 0.001$) with no significant lack of fit. The age effect

Tab. 1. The results of recovery and precision studies for serum hydroxyproline assay.

Recovery study

Control pool = 122.5 μmol/l Mean value of four replicates

Spiked value (μmol/l)	Assay value (μmol/l)	Recovery (%)
76.3	174.8	68.5
152.7	248.1	82.3
305.3	401.5	91.4
610.7	638.2	84.4
		$\bar{x}$ = 81.7

Within run precision

	Group A	Group B	Group C	Group D
Mean value (μmol/l)	129.8	209.2	238.2	3.9
SD (μmol/l)	7.6	11.5	6.9	7.6
CV (%)	5.9	5.5	2.9	1.9
N	10	10	10	10

Day-to-day precision

	Control I	Control II	Pool A	Pool B
Mean value (μmol/l)	84.0	116.8	130.5	412.2
SD (μmol/l)	6.9	13.0	13.0	43.5
CV (%)	8.2	11.1	10	10.6
N	21	21	20	20

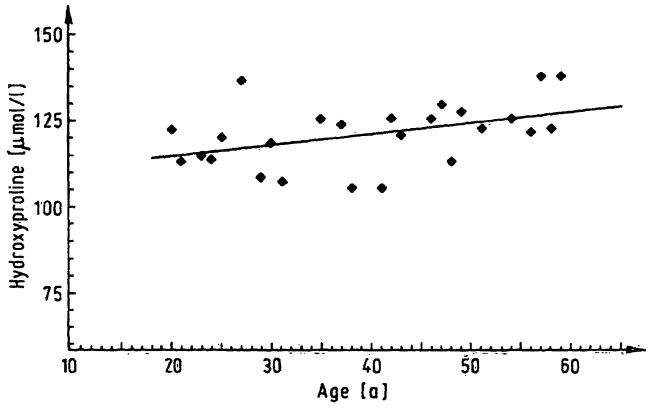

Fig. 1. The relationship between serum hydroxyproline mean values and age for males, y (hydroxyproline) = 108.4927 + 0.31618 (age).

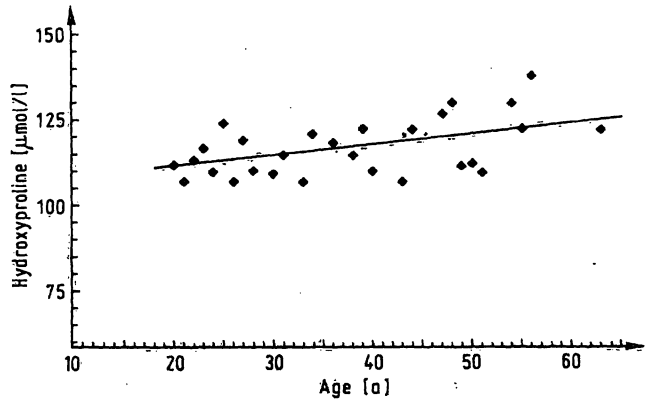

Fig. 2. The relationship between serum hydroxyproline mean values and age for females, y (hydroxyproline) = 105.3675 + 0.31618 (age).

($p < 0.001$) is linear and homogeneous among sexes. The effect due to sex (sexes adjusted to a common age) approaches significance ($p = 0.08$). The values were fitted by the model y = 108.4927−3.1253 (sex) +0.31618 (age) where sex = 1 if female and 0 if male.

A similar sex effect has been reported for urinary hydroxyproline (2). This effect was attributed to the greater collagen mass in males compared with females.

Age effects occurring during the adolescent "growth spurt" have been reported for urinary hydroxyproline (2). Urinary hydroxyproline increases during adolescence and decreases at maturity. Adult age effects have not been reported.

Total hydroxyproline was assayed in the sera of 22 patients with *Paget*'s disease who were attending the metabolic bone disease clinic at the Jewish Hospital of St. Louis (tab. 2). Most were being treated with either salmon calcitonin or diphosphonate and were preprandial. Their disease was in different states of control at the time these sera were obtained. The ages of most of these patients fall outside our normal

Tab. 2. The results of the measurement of hydroxyproline in the serum of *Paget*'s disease patients and the corresponding predicted normal values based on the patient's age and sex.

Subject no.	Age	Sex	Observed value hydroxyproline (μmol/l)	Predicted normal value (μmol/l)	Difference (μmol/l)
1 RM	67	♂	174.0	129.7	44.4
2 ZM	66	♂	251.9	129.4	122.5
3 PM	61	♂	134.4	127.8	6.6
4 BM	71	♂	160.3	130.9	29.4
5 HM	70	♂	142.0	130.6	11.4
6 KM	70	♂	135.9	130.6	5.3
7 BM	62	♂	190.8	128.1	62.7
8 HM	78	♂	128.2	133.2	−4.9
9 CM	59	♂	151.1	127.1	24.0
10 SM	73	♀	134.4	128.4	5.9
11 RM	80	♂	138.9	133.8	5.1
12 WM	56	♂	125.2	126.2	−1.0
13 KM	69	♂	137.4	130.3	7.1
14 RM	66	♂	164.9	129.4	35.5
15 PM	62	♂	184.7	128.1	56.6
16 WM	55	♂	137.4	125.9	11.5
17 RF	69	♀	122.1	127.2	−5.0
18 TF	55	♀	206.1	122.8	83.4
19 KF	73	♀	134.4	128.4	5.9
20 SF	74	♀	143.5	128.8	14.7
21 GF	75	♀	160.3	129.1	31.2
22 KF	59	♀	142.0	124.0	18.0

range study. The results were analyzed by predicting their normal value based on their age and sex using the model developed for normals. The predicted normal value was subtracted from the measured value. The differences were significantly greater than zero using a simple T test (5) ($p < 0.001$) indicating the expected elevation (average of +25.,9 μmol/l; standard error of 6.7 μmol/l) in serum hydroxyproline for this group of patients with a disease characterized by accelerated bone remodeling and enhanced collagen degradation.

A correlation between serum alkaline phosphatase (6) and serum hydroxyproline was also observed for the *Paget*'s patients. The correlation coefficient was 0.91 ($p < 0.01$) and the regression line is shown in figure 3.

We conclude that this assay of hydroxyproline in 12 h fasted serum will prove useful for evaluating diseases involving collagen if age and sex are taken into consideration.

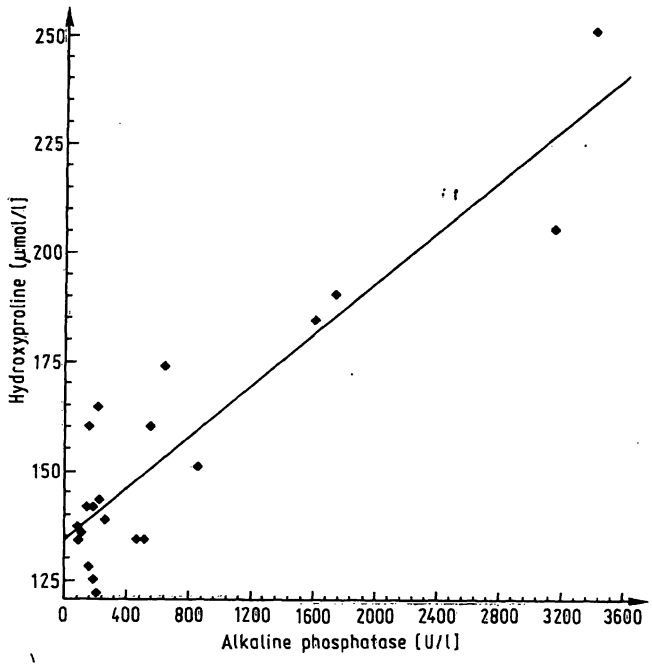

Fig. 3. The correlation between serum hydroxyproline and serum alkaline phosphatase in *Paget*'s disease patients (r = 0.91).

References

1. Udenfriend, S. (1966) Science *152*, 1335–1340.
2. Leroy, E. C. (1967) Adv. Clin. Chem. *10*, 213–253.
3. Blumenkrantz, N. & Asboe-Hansen, G. (1974) Clin. Biochem. *7*, 251–257.
4. Graybill, F. A. (1961) An Introduction to Linear Statistical Models *1*, McGraw-Hill Inc., New York, New York.
5. Ostle, B. (1963) Statistics in Research, Iowa State University Press, Ames, Iowa.
6. Morgenstern, S., Kessler, G., Auerbach, J., Flor, R. V. & Klein, B. (1965) Clin. Chem. *11*, 889–897.

Terry J. Gilbertson, Ph. D.
The Upjohn Company
Kalamazoo, Michigan 49001
U.S.A.